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**14. ABSTRACT**

The overall goal of this research is to preserve vision of patients recovering from severe facial burns by providing an improved method to reduce development of corneal defects, inflammation, infection and opacification. **Scope:** To further improve and understand the properties of the degradation-resistant crosslinked amniotic membranes for treating cornea of burn patients that were produced in Year 1. **Major findings**: Established that 2 and 3 layer crosslinked amniotic membrane composites are more resistant to enzymatic degradation and thus expected to be more lasting on patients' eyes, that chemical and photo-initiated crosslinking of amnion proteins are associated with the loss of TGF-®, a cytokine tightly associated with crosslinked proteins in amnion, and that crosslinked amnion can be photobonded to cornea ex vivo thereby providing an alternative for suturing these degradation-resistant membranes to the ocular surface. An amnion-covered contact lens was constructed and photobonded to ex vivo cornea as an approach to supplying hydration to burn patients eyes.

**15. SUBJECT TERMS**
ectropion, cornea, burn scars, amniotic membrane, protein crosslinks, photomedicine, photochemistry, proteinases

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**INTRODUCTION:**

The overall goal of this research is to improve the visual outcomes as well as the quality of life for burn patients during the acute and convalescent phases of their rehabilitation. Scarring from second and third degree facial burns, and from subsequent skin grafts, causes the tissues involved to contract and, if significant enough, the patient is left unable to blink or close their eyes. This results in desiccation of the ocular surface, breakdown of the cornea's defense mechanisms and subsequent events that may lead to cornea opacification and the need for cornea transplant. Currently these patients receive frequent application of topical lubricants and anti-inflammatory medications, an imperfect solution that requires very frequent administration of drops which keeps the wounded warrior at the bedside and can slow down other rehabilitation services that are remote to the inpatient ward. Amniotic membrane (AM) transplantation to the ocular surface can assist in the maintenance of the ocular surface of these patients. However, commercially available membrane is not only very expensive, but enzymes on the inflamed ocular surface degrade and destroy the AM very rapidly; this occurs in one day compared to two weeks in non-burn patients. In this project, our major goal is to stabilize the amniotic membrane by crosslinking it's constitutive proteins before applying it to the patient's eye. We will determine the crosslinking method that most effectively decreases the rate of enzymatic degradation of AM while preserving the beneficial anti-inflammatory and pro-healing factors in the membrane. In addition, we will evaluate photobonding as a sutureless, glueless alternative to sutures for attaching AM to cornea. We will also test an approach that combines AM with a hydrogel material to increase the ability of the amnion to hydrate the cornea. These studies will employ a rabbit model of eye inflammation.

**BODY:**

This Grant Agreement is a joint proposal with COL Anthony J. Johnson, MD, PI on Grant Agreement W81XWH-09-2-0069. The Statement of Work includes tasks to be carried at both the Massachusetts General Hospital and the Brooke Army Medical Center (BAMC). An objective of the PRMRP was to enhance the translation of results from bench research to clinical problems and, in turn, to facilitate learning fundamental information from medical applications. In this project period, Drs. Kochevar and Johnson discussed by phone the animal model being developed for ectropion (eye inflammation in a rabbit), the methods for assessing inflammatory mediators and growth factors, and updated each other on progress. Dr. Kochevar visited Dr. Johnson's facilities at BAMC in December 2010.

Major tasks for Year 2 of this project were to: 1) Determine whether a multilayer composite of amnion undergoes slower proteolytic degradation than a monolayer membrane, 2) Establish an animal model for ectropion, 3) Identify the protein crosslinking method that produces the least reduction in beneficial healing factors in amnion, 4) Determine whether photobonding of crosslinked amnion to cornea is feasible, and 5) Construct an amnion-covered hydrogel bandage. The results in this Year 2 annual report follow the order of the specific aims of the grant proposal.

**Specific aim 1.b. Determine whether a multilayer composite of amnion retards the rate of proteolytic degradation in vitro.**

The studies during Year 1 identified two methods for crosslinking amniotic membrane (AM) proteins that inhibited enzymatic degradation of the amnion. Those studies were carried out using single layers of amnion (~50 μm thick). Another approach to stabilizing amnion against

enzymatic degradation is to increase the thickness of the membrane by sealing together a stack of AM layers. *The hypothesis is that the protein in the center of this thicker membrane would be less accessible to proteolytic enzymes; consequently, the time required to degrade the amnion would increase and this approach might lead to an material with a longer use time for treatment of corneas of burn patients with severe periorbital scarring.*

To test this hypothesis, we evaluated multilayer AM constructs in which the layers were sealed together using the chemical crosslinking and photo-crosslinking methods reported in Year 1. The chemical crosslinking method was treatment with a carbodiimide. The photo-crosslinking method utilized Rose Bengal dye and green (532 nm) light. Using both of these methods, we tested whether protein-protein crosslinks formed both between amnion layers and crosslinks within the AM increase the enzymatic degradation time.

**1.b.1. Amniotic membrane: preparation and enzymatic degradation**

The methods used to harvest AM, to treat the amnion with proteolytic enzymes and to measure the extent of enzymatic degradation of proteins in the membrane were described in detail in the Year 1 annual report. These procedures are only briefly described here.

Human placentas from scheduled caesarian section deliveries at Massachusetts General Hospital were obtained with the approval of the Institutional Review Board of the Partners Healthcare System. The AM was peeled away from the chorion and epithelial cells were removed by treatment with trypsin and scraping. The segments were stored at -80˚C and immediately prior to use were defrosted and rinsed thoroughly.

Enzymatic degradation of AM was carried out by incubating samples at 37°C with collagenase (Type I from Clostridium histolyticum). The negative control contained only collagenase (no AM). Multiple enzymatic cleavages of the membrane proteins degrade the AM and release peptide fragments containing free amino groups. We measured the level of these peptide free amino groups in the medium using a fluorescence assay (1) to determine the extent of degradation of collagen in AM. The percent proteolytic degradation of AM proteins in treated samples was compared to the level in untreated controls.

**1.b.2. Preparation of multilayer amnion composites and assessment of proteolytic degradation**

*1.b.2.i. Chemical crosslinking with carbodiimide to produce multilayer membranes*

The results in Year 1 indicated that treating AM with 20 mM carbodiimide [1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride] for 24 h lead to nearly complete inhibition of collagenase degradation. Although inhibition of proteolytic degradation was a goal, the fully crosslinked membrane was stiffer than untreated AM. It was not sufficiently pliable to conform to the shape of a cornea, and thus could not be used for treating corneas of burn patients. Consequently, we first carried out experiments to identify carbodiimide concentration and treatment conditions that did not greatly increase the AM stiffness.

Amnion segments were treated with carbodiimide and NHS (N-hydroxsuccinimide) in 0.05 M MES (N-morpholino)-ethansulfonic acid) buffer as adapted from the procedure of Ma et al., (2). The carbodiimide concentration was varied (5-10 mM) and the treatment time was varied (1-30 min). The results in Fig. 1A indicate that increasing the reaction time decreased the percent proteolytic degradation compared to the untreated control. Treatment with 5 mM carbodiimide for only 5 min afforded ~ 50% protection from collagenase digestion. This membrane was only slightly less pliable than untreated AM. We realize that quantification of the stiffness of

crosslinked membranes is needed to select the appropriate modified AM for the goals of this study. Although these measurements were not part of the original proposal, we will soon be able quantify physical properties related to membrane stiffness because we will have access to a new tensiometer that can be used to measure small amnion samples.

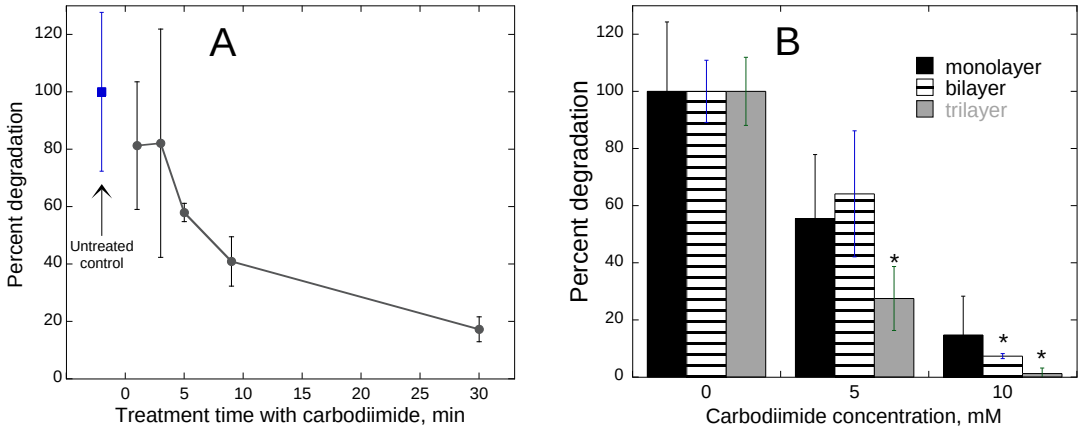


Figure 1. Comparison of proteolytic degradation of crosslinked multi-layer amnion membranes with single layer amnion. The percent degradation of amnion proteins by bacterial collagenase was measured from the release of peptide fragments. **(A)** Influence of reaction time with 5 mM carbodiimide on degradation of monolayer amnion. (**B**) Comparison of the susceptibility of mono-, bi- and trilayer amnion composites to proteolytic degradation after crosslinking with 5 and 10 mM carbodiimide. * indicates $p < 0.05$ compared to monolayer amnion treated with the same carbodiimide concentration

Next, composites of 2 and 3 amnion layers were constructed. To bond together multiple layers of AM, the membrane surfaces must be in close contact during the treatment for formation of molecular crosslinks between proteins on the surfaces of the two layers. This was accomplished by placing 2 or 3 circular discs of amnion (13 mm diameter) between fine mesh circular brass screens and securing the circumference only. This arrangement allowed the reagents to diffuse into the membranes through the screens while keeping the layers in tight contact. The AM layers were stacked with each stromal surface in contact with the basement membrane surface of the next AM layer.

Amnion discs were dried and weighed (0.8 – 1.3 mg each), then assembled and treated (in triplicate) as mono, bi or tri-layers with 5 or 10 mM carbodiimide and in NHS and MES buffer for 1 h at room temperature. Control samples were treated with MES buffer only. After the reaction period, the AM composite was thoroughly washed in distilled water to remove excess reagents and byproducts. The layers of the control, untreated AM discs separated whereas the carbodiimide-treated constructs remained stacked indicating that crosslinking between the layers had occurred. Each sample was then digested with bacterial collagenase for 20 h and the release of peptide fragments containing free amino groups was measured as described in section 1.b.1.

The percent degradation in the layered composites compared to the control (not treated with carbodiimide) is shown in Fig. 1B. The trilayer membrane crosslinked with 5 mM carbodiimide was more resistant to proteolytic degradation than the similarly-treated monolayer ($p < 0.05$), consistent with the hypothesis that the thicker membrane protects AM proteins from proteolytic degradation. This result is supported further by the results obtained using 10 mM carbodiimide;

both the bi- and trilayer membranes showed less proteolytic degradation than the monolayer membrane ($p < 0.05$).

**Summary/Conclusion** Crosslinking bi- and tri-layer AM composites with carbodiimide retards enzymatic degradation compared to monolayer membranes suggesting that these materials may be superior for protecting corneas of ectropion eyes.

*1.b.2.ii. Photochemical crosslinking with Rose Bengal to produce multilayer amniotic membrane composites*

For effective bonding using a photosensitization method, sufficient light must reach the interface between the two layers to activate the dye leading to protein-protein crosslinks between layers. Thus, initial studies were carried out to measure the amount of 532 nm (green) light transmitted by the RB-stained amnion. Amnion was stained for varying times with either 0.05% or 0.10% RB. The results (Fig. 2) indicate that even short staining times (5 min) were sufficient for the AM to absorb RB. Since an absorbance of 1.0 at 532 nm corresponds to absorption of 90% of the light, we chose 5 min treatment with 0.10% RB as our standard RB staining treatment.

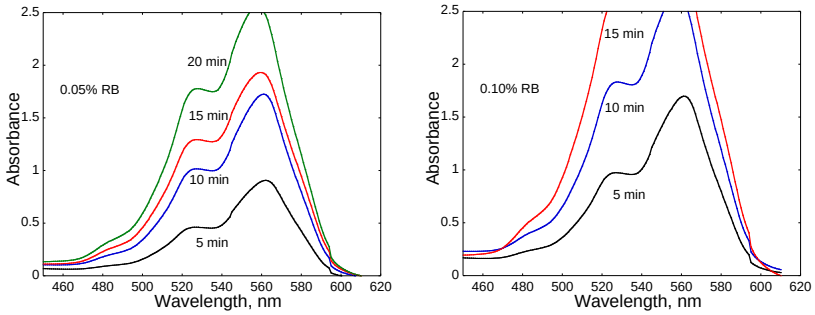


Figure 2. Absorption spectra of RB (0.05 and 0.10% in PBS) after application to amniotic membrane for varying times. Membranes were then washed before spectra were taken.

Next, we investigated whether bonding two or three amnion layers together with RB + green light produced a composite membrane that was less susceptible to degradation by bacterial collagenase. For this study, the RB treatment was 0.1% RB for 5 min prior to irradiation and the 532 nm fluence was 50 J/cm$^2$. The experimental groups were: 1) monolayer amnion treated with RB and 532 nm, 2) two-layers of amnion stained with RB and irradiated sequentially, and 3) three layers amnion stained and irradiated in a sequential manner. Control groups were untreated amnion and monolayer of amnion treated with RB but not irradiated. Each sample was incubated with bacterial collagenase for 20 h as described in 1.b.1. The results shown in Fig. 3 indicate that bilayer photocrosslinked composites are ~20% more resistant to collagenase Next, we investigated whether bonding two or three amnion layers together with RB + green light produced a composite membrane that was less susceptible to degradation by bacterial collagenase. For this study, the RB treatment was 0.1% RB for 5 min prior to irradiation and the 532 nm fluence was 50 J/cm$^2$. The experimental groups were: 1) monolayer amnion treated with RB and 532 nm, 2) two-layers of amnion stained with RB and irradiated sequentially, and 3) three layers amnion stained and irradiated in a sequential manner. Control groups were untreated amnion and monolayer of amnion treated with RB but not irradiated. Each sample was incubated with bacterial collagenase for 20 h as described in 1.b.1. The results shown in Figure 3 indicate that bilayer photocrosslinked composites are ~20% more resistant to collagenase digestion than monolayers and that trilayers are almost totally resistant to enzymatic degradation.

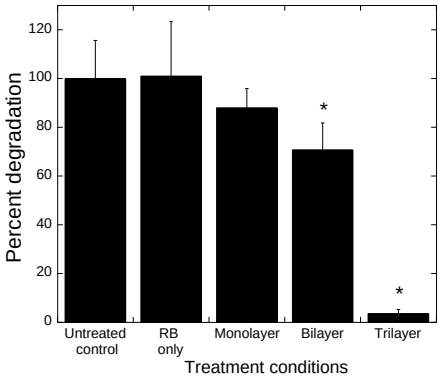


Figure 3. Protective effect of photo-crosslinking amnion layers against enzymatic degradation. Amnion layers (2 or 3) were photobonded together and photocrosslinked using Rose Bengal and 532 nm light. The percent proteolytic degradation was assessed by incubation with bacterial collagenase for 20 h. * indicates $p < 0.05$ compared to monolayer.

**Summary/conclusions** Photo-crosslinking 2 and 3 layer amnion composites decreases the susceptibility of amnion to proteolytic degradation compared to amnion monolayers. These results are similar to those obtained after carbodiimide crosslinking. The choice between these two methods for producing a membrane for protection of cornea of inflamed eyes requires measurements of the stiffness of the composites as well as information on retention of pro-healing/anti-inflammatory proteins during the crosslinking.

**Specific aim 1.c. Assess biochemical and structural alterations in cornea of rabbit model for ectropion.** Create ectropion by blepharoplasty in New Zealand white rabbits. Assess epithelial defects and corneal ulcers. Measure inflammatory cytokines and proteolytic enzymes.

**1.c.1. Create animal model:**
The animal study protocol was approved on April 16, 2010 by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of rabbits and was approved by the Brooke Army Medical Center IACUC ((IACUC Protocol Number A-2009-02). Dr. Johnson's report details the development of this model.

**1.c.2. Measure inflammatory cytokine and proteolytic enzymes.**
Matrix metalloproteinases (MMPs) in tears of inflamed eyes are responsible for degrading collagen and other corneal proteins as well as amnion proteins. We selected an assay that uses a fluorescence readout for assaying MMPs because of its high sensitivity, ability to measure both MMP-2 and MMP-9 and its simplicity and speed (EnzChek® Gelatinase/collagenase assay kit, Molecular Probes). A gelatin substrate is used that is heavily labeled with fluorescein; under these conditions the fluorescence is quenched due to intra-molecular processes. When an MMP cleaves the substrate protein, the fluorescein molecules are able to fluoresce when excited at 485 nm; emission is at 530 nm.

We have established this assay in the lab and verified that the sensitivity is $2 \times 10^{-3}$ U/ml (7 ng protein/ml) using Clostridium histolyticum collagenase. According to the manufacturer, for human MMP-2 proteolysis of the fluorescent substrate the assay detection limit is $3 \times 10^{-4}$ U/ml. This assay can be used to quantify human, rabbit and mouse MMPs.

Inflammatory cytokines such as TNF-$\alpha$ and IL-1 are generally measured by commercially available ELISA assay kits. ELISA kits specific for rabbit cytokines are not common although some commercially available ELISA kits for human cytokines are reported to cross react with

rabbit cytokines. We will test these available assays, if the ectropion model is developed in the rabbit. Mouse cytokine ELISA kits are commercially available, if the ectropion model is developed in this species.

**Specific aim 1.e. Identify the protein crosslinking method that has causes the least reduction in anti-inflammatory and healing factors in amnion.**

Amniotic membrane contains several growth factors (EGF, TGF-α, KGF, HGF, bFGF) and cytokines (TGF-β1,TGF-β2, others) believed to contribute to the pro-healing and anti-inflammatory properties of aminon (3, 4). Growth factors and cytokines are produced mainly by the epithelial cells of amnion and many are stored in the stroma. We chose to test the effect of our amnion protein crosslinking methods on the cytokine most likely to be affected by these crosslinking treatments, namely, TGF-β1. This factor is stored in the stroma tightly bound to extracellular matrix proteins. Thus, crosslinking chemistry is likely to form links between the matrix proteins and TGF-β1, thus decreasing its bioavailability.

*1.e.1. Extraction of inflammatory mediators/healing factors from amnion.*
Although immunofluorescence has be used to detect the presence of TGF-β1 and other growth factors, quantification requires extraction of proteins from amnion. Amniotic membrane segments (1.2-2.4 mg) were manually homogenized in a liquid nitrogen-cooled 2.75-inch diameter porcelain mortar and pestle. During homogenization 3 mL PBS was slowly added. Upon completion, 30 µL of protease inhibitor cocktail (P-8340, Sigma) was added and the suspension was transferred to Eppendorf tubes, and centrifuged at 14,000 g for 15 min. The supernatant was concentrated using Millipore-Amicon Ultra Centrifugal Filters (0.5 mL, 3K membrane). Protein concentration of the homogenate and concentrated solution were measured (DC BioRad Protein Assay). Typically, from a 20 mg segment of dried amnion, the concentrated solution contained 0.25 mg soluble protein.

*1.e.2. Measurement of TGF-β1*
Pilot experiments were carried out to measure TGF-β1 by SDS-PAGE separation and western blotting as this method might be able to quantify more than one mediator using the same analysis. However, the levels of TGF-β1 were not detectable by this method. Consequently, TGF-β1 was assayed using a commercial ELISA kit (TGF-β1 $E_{max}$® ImmunoAssay Systems, Promega). The valid range for this assay was 5 to 1000 pg/ml. These measurements were converted in to the more meaningful values of pg of TGF-β1 per mg of amniotic membrane.

TGF-β1 in tissue is crosslinked in a storage complex with latent TGF-β-binding protein. Acid activation releases the TGF-β1, thus making it available for measurement. We tried two approaches to acid activation. In the first experiments TGF-β1 was assayed following the manufacturer's procedure, in which acid activation (pH 6.5 for 15 min) was after extraction of proteins from the amnion (crosslinked and uncrosslinked). However, the TGF-β1 released did not correlate with the extent of crosslinking (as determined by proteolytic degradation). Consequently, we changed the protocol and acid activated the TGF-β1 release during the extraction of protein from the amnion.**specific procedure** This extraction procedure was used for the studies described below.

*1.e.2.i. Effect of crosslinking with carbodiimide on TGF-β content.*

Amniotic membrane segments were treated under a variety of conditions (carbodiimide concentration, reaction time) to examine conditions that might produce protein crosslinking with minimal loss of TGF-β1. In the experiment shown in Fig. 4, the concentrations of carbodiimide were 6 to 20 mM (in MES buffer) and the reaction time was 5 to 60 min. The control was amnion treated for 1 h in MES buffer only. Samples of the treated amnion (~1 mg each) were taken from each treatment group to measure the extent of crosslinking by proteolytic degradation. The TGF-β1 was assayed by ELISA as described above.

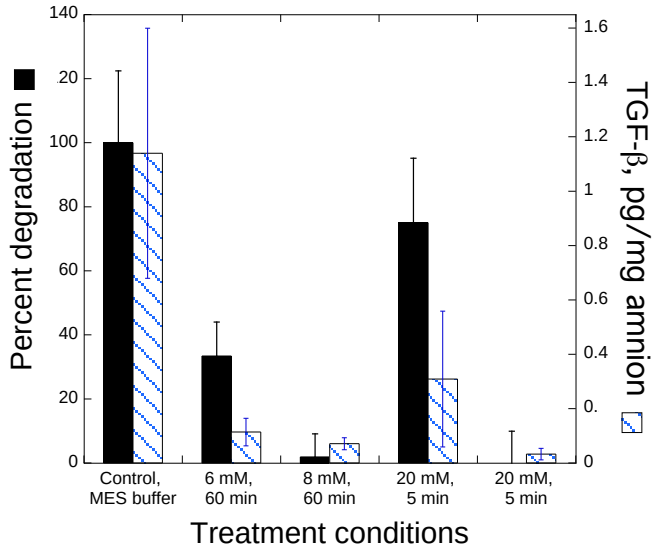


Figure 4. Effect of crosslinking amnion proteins with carbodiimide on the level of TGF-β. The percent proteolytic degradation of treated and control amnion is shown as black bars. The level of TGF-β was measured on the same samples by ELISA (blue stripped bars).

The results in Fig. 4 show that the crosslinking treatments with carbodiimde produced amnion that was resistant to proteolytic degradation (black bars). Treatment with 8 mM carbodiimide for 60 min and with 20 mM for 5 min completely blocked enzymatic degradation, consistent with our previous results (e.g., Fig. 1B). A lower carbodiimide concentration (6 mM, 60 min) or shorter reaction time (20 mM, 5 min) inhibited degradation to 31% and 72%, respectively. Importantly, all the treatments with carbodiimide markedly or completely blocked TGF-β1 release (stripped bars). Only treatment with 20 mM carbodiimide for 5 min resulted in greater than 10% of the control level of TGF-β1. This result indicates that the carbodiimide-treated amnion, although it is resistant to enzymatic degradation, does not retain the native level of a potentially beneficial cytokine, TGF-β1.

*1.e.2.i. Crosslinking with Rose Bengal + green light.* The types of molecular crosslinks formed between amnion proteins differ between amnion treated with carbodiimide treatment versus RB photo-crosslinking. Thus, it was important to determine whether RB photo-crosslinking affected the level of TGF-β1 more, less or the same as crosslinking with carbodiimide. Amnion samples were stained with 0.1% RB for 5 min, then exposed to fluences of 532 nm between 2 and 50 J/cm$^2$. The effect of these treatments on degradation of amnion proteins by bacterial collagenase was measured. The results from one experiment (of three) using 532 nm fluences of 5 and 50 J/cm$^2$ are shown in Fig. 5 (black bars). Photo-crosslinking inhibited collagenase degradation to 51% using 5 J/cm$^2$ and to 42% using 50 J/cm$^2$ compared to the untreated control level. The TGF-β1 levels (striped bars) in the amnion treated with RB and 532 nm decreased. At the higher fluence (50 J/cm$^2$), TGF-β1 release was entirely blocked. At the lower fluence,

which decreased degradation by ~50%, almost 30% of the TGF-β1 was still available.

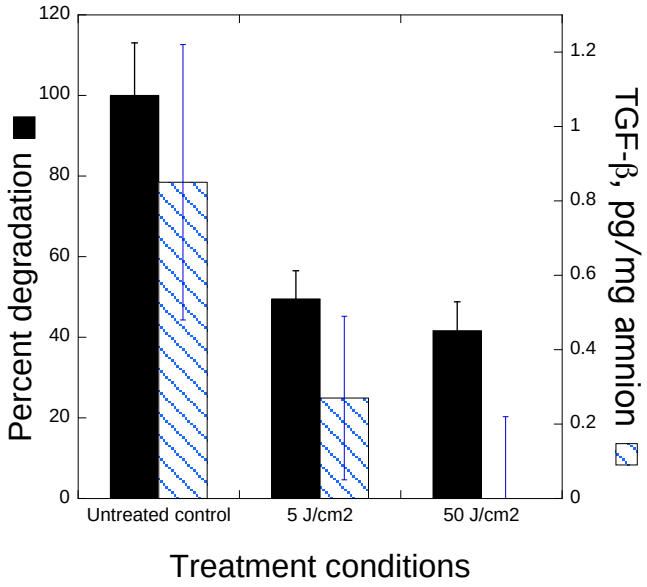


Figure 5. Effect of photo-crosslinking amnion proteins using Rose Bengal and green light on the level of TGF-β. Proteolytic degradation of amnion was measured (black bars). The level of TGF-β was measured on the same samples by ELISA (blue stripped bars).

**Summary/Conclusions**

Crosslinking of amnion proteins with carbodiimide or by RB photosensitization significantly reduces the amnion content of TGF-β1, a factor involved in wound healing. Thus, the crosslinking conditions that produce degradation resistant amnion also cause loss of this cytokine. Further work is needed to determine whether cytokines and growth factors that do not associate with the stromal proteins are preserved during crosslinking amnion proteins. This result suggests that stabilizing amniotic membrane against proteolytic degradation by crosslinking alters at least one of the beneficial properties of this biomaterial for protecting ectropion eyes.

**Specific aim 1.f.i. Evaluate the healing properties of crosslinked amnion on the rabbit ectropion model.**

We will measure matrix metalloproteinases (MMPs) in tears of ectropion model eyes when they are available. We selected the MMP level as an outcome because these enzymes are responsible for degrading collagen and other amnion proteins, leading to the short useful lifetime of AM for protecting corneas of ectropion patients. The fluorescent peptide substrate assay described in section 1.c.2 will be used. For cytokine measurements, commercially available ELISA kits will be used with the limitations descried in section 1.c.2.

**Specific aim 2. To determine whether a photoactivated method for bonding amnion to cornea provides a rational alternative to suturing.**

The crosslinked, degradation-resistant amnion that is being developed in this project could be placed on the inflamed eye in a Prokera®–like device or be sutured to the cornea as is done in amniotic membrane transplantation. However we proposed and, have now tested, an alternative method for securing the crosslinked amnion to cornea, namely, photobonding the membrane to the cornea. In previous studies (not part of this project) we demonstrated that native AM (non-

crosslinked) is tightly bonded to normal cornea using RB and green light. This process photo-crosslinks proteins between the two surfaces to produce a molecular level seal (manuscript submitted). Since photobonding AM to cornea does not require an additional ring to hold the amnion in place (such as used for the Prokera® device) and allows trimming of the amnion to the size and shape needed for each application, this approach has potential advantages.

These experiments were designed to determine whether pre-crosslinked amnion could be photobonded to de-epithelialized cornea. Since both pre-crosslinking and photobonding involve certain protein sites, there was concern that pre-crosslinking might destroy all the potential sites before the amnion was photobonded to cornea.

Our previous studies indicated that the most reproducible system for testing the strength of amnion bonding to cornea was to seal the AM over a full thickness V-shaped incision in cornea (Fig. 6). *These studies test the hypothesis that AM, which has been made resistant to enzymatic degradation by crosslinking using the two methods described above, can be sealed to the cornea surface by photo-crosslinking.*

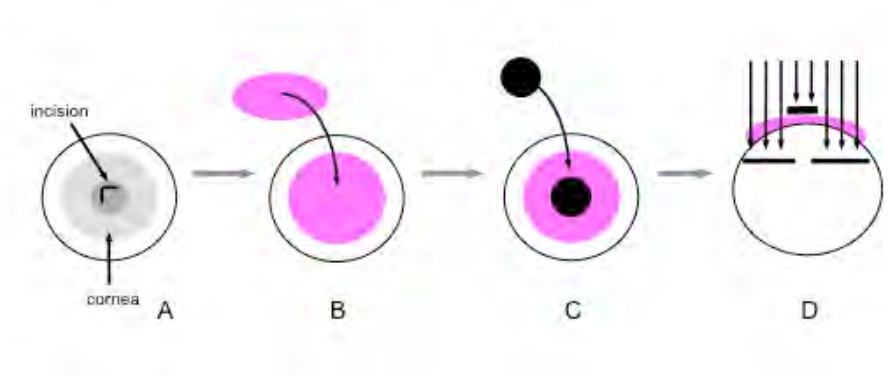


Figure 6. Schematic drawing of technique for sealing a penetrating cornea wound with a light-activated process. (**A**) An incision is made in the central cornea. (**B**) A Rose Bengal-stained pre-crosslinked amnion patch is placed over the cornea. (**C**) An opaque disc is placed over the pupil. (**D**) The amnion-covered cornea is irradiated with green (532 nm) laser light (side view).

**2.a. Crosslinking amnion proteins with carbodiimide prior to bonding to cornea.** Discs of amnion, 13-mm diameter, were treated in 10 mM EDC or 20 mM EDC as described in 1.b.2.i. Controls were treated for the same period in NHS and MES buffer alone. After washing out excess reagents and byproducts, the membranes were dried, stained with 0.1% RB for 5 min, then placed over the V-shaped full thickness wound in the central cornea of de-epithelialized ex vivo rabbit eyes (Fig. 6). A 4-mm pupil block was placed and each eye was irradiated with 532 nm light (0.25 W/cm$^2$). Three fluences were used: 50, 100 and 150 J/cm$^2$.

The bonding strength was measured by infusing a saline solution containing a blue dye into the anterior chamber and measuring the pressure we have done previously (5). The maximum pressure attained before leakage from under the amnion is observed is termed the leak intraocular pressure ($IOP_L$) and is reports on the bonding strength.

In the untreated control (0 mM carbodiimide) the $IOP_L$ increased as the fluence increased from ~45 mm Hg using 50 J/cm$^2$ to ~180 mm Hg using 150 J/cm$^2$ (Fig. 7A). Membranes that were pre-crosslinked with 10 mM carbodiimide showed the same $IOP_L$ as the untreated control membranes when exposed to 50 and 100 J/cm$^2$ indicating that pre-crosslinking did not hinder bonding of AM to the cornea surface. Thus,10 mM carbodiimide-treated amnion, which is highly resistant to enzymatic degradation, can be photobonded to cornea. *This result supports the*

*hypothesis that a crosslinked, degradation-resistant AM can be securely sealed to cornea by photobonding.*

Pre-crosslinking with 20 mM carbodiimide decreased the bonding strength of amnion to cornea, as seen from the lower $IOP_L$ values. The poor bonding strength resulted from the relative stiffness of the AM produced by extensive protein crosslinking during pre-treatment with carbodiimide. These membranes were not sufficiently flexible to place in tight contact over the curved surface of the cornea. Consequently, leakage readily occurred at low IOP.

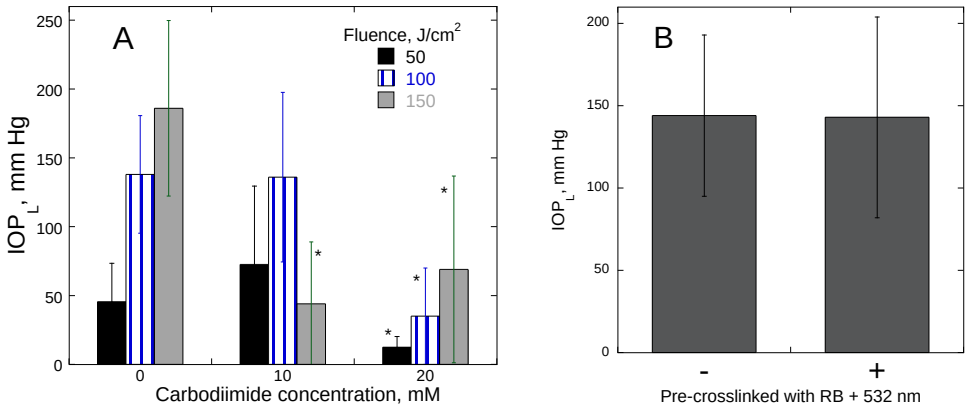


Figure 7. Influence of pre-crosslinking amniotic membrane proteins on the photobonding of amnion over a full thickness incision in the corneal surface. Photobonding used Rose Bengal dye and green light. The strength of bonding between amnion and cornea was measured as the anterior chamber pressure that causes disruption of the bond ($IOP_L$). (**A**) Amnion pre-crosslinked with carbodiimide, then photobonded to cornea. Asterisk indicates $p<0.05$ compared to control, non-crosslinked amnion at the same photobonding fluence (**B**) Amnion was pre-photocrosslinked by RB photosensitization, then photobonded.

**2.b. Crosslinking amnion proteins with Rose Bengal and green light prior to bonding to cornea.** The influence of pre-crosslinking of AM proteins with Rose Bengal and green (532 nm) light on photobonding the membrane to cornea was tested using the same assay system as described for carbodiimide crosslinking. The 13-mm discs of amnion were stained with RB for 5 min, then irradiated with 100 J/cm$^2$ 532 nm. This membrane was then placed on the cornea and photobonded as described above for carbodiimide crosslinking. Figure 7B shows the results from a comparison of AM not crosslinked (left bar) with AM crosslinked with RB/green light prior to photobonding. Clearly, pre-treatment with RB/green light did not alter the ability of photobonding to seal AM over the corneal wound.

**Summary/Conclusions** These results indicate that pre-crosslinked AM that is resistant to enzymatic degradation can be tightly sealed to the cornea surface. This technique may provide a viable alternative to sutures for securing degradation-resistant amnion to cornea for treatment of cornea of ectropion eyes.

**Specific aim 3.a. Construct amnion-covered hydrogel bandage.** Use photochemical crosslinking to seal hydrophilic material within an amnion covering.

Currently corneal hydration for patients with cicatricial ectropion is maintained by frequent administration of artificial tears. We have tested an alternative approach, which involves photo-crosslinking of amnion proteins. Amnion, by itself, does not supply moisture to the cornea. Consequently, we have encapsulated a hydrogel contact lens within an amnion capsule to provide a source of moisture for the cornea. Bonding this construct to the cornea using a light-activated process would keep the contact lens construct in place and prevent bacterial infection under the contact lens. In Year 2 our task was to fabricate the amnion-encapsulated hydrogel lens.

A hydrophilic hydrogel contact lens (1 DAY ACUVUE - TRUeye) was used because its high water content allows for flexibility and efficient oxygen diffusion to the ocular surface. To encapsulate the lens, a circular disc of amnion (18 mm diameter) was placed on a hard sphere with approximately the curvature of a rabbit cornea. The stromal surface of the de-epithelialized amnion was placed in contact with the sphere (Step 1 in Figure 8). The contact lens was then placed on this surface (Step 2). A 15-mm diameter circular piece of amnion was stained with RB on the stromal surface around the perimeter (~2 mm band) (0.1% RB/5 min) and then placed on over the contact lens such that the two amnion surfaces were in contact (Step 3). The composite was sealed by irradiating this composite with green light at 532 nm (200 J/cm$^2$ at 0.20 W/cm$^2$). The light activates the RB dye around the perimeter of the amnion thereby photocrosslinking proteins between the two amnion surfaces (Step 4).

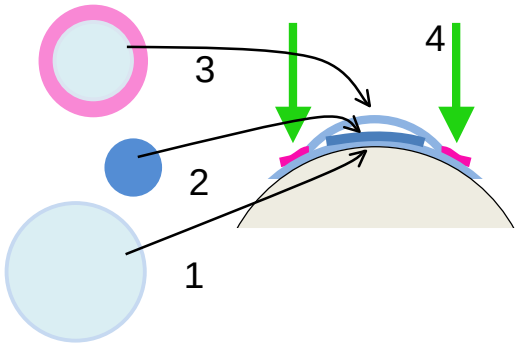


Figure 8. Encapsulating a hydrophilic contact lens with amniotic membrane. (1) Amnion disc (18 mm) placed on hard sphere. (2) Contact lens placed on amnion disc. (3) Amnion (15 mm) with RB-stained perimeter placed over lens and lower amnion. (4) RB-stained amnion is irradiated with 532 nm (green light) to seal the two amnion layers together.

To test the integrity of the seal around the contact lens, a 25-gauge needle was inserted into the chamber containing the lens, and blue dye solution was slowly pumped in. The pressure within the chamber was monitored and reached values between 2 and 110 mm Hg (n = 18) before leakage occurred . The great variability in the pressure causing leakage appeared to be caused by small wrinkles in the membrane that did not allow tight contact between the two amnion surfaces during the irradiation. Modifying the technique did not significantly imp

Preliminary tests were carried out to determine whether the amnion-encapsulated contact lens construct would bond to a cornea ex vivo. The bottom amnion-covered surface of the construct was stained with RB (0.1% for 5 min). This was then placed on the de-epithilialized cornea surface of an ex vivo rabbit eye containing a V-shaped incision as described in section 2.a. The contact lens and cornea were irradiated from above (150 J/cm$^2$). The strength of the bond between the construct and the cornea was assessed using the technique described in section 2.a. Measurements of the leak intraocular pressure on 5 eyes gave a value of 87 ± 41 mm Hg.

This pilot study result indicates that the concept of bonding a hydrating contact lens to the cornea is potentially feasible.

**Summary/Conclusion** Construction of an amnion-encapsulated hydrophilic lens is feasible using crosslinking methods to seal the capsule. Preliminary studies suggest that these constructs can be bonded to the cornea. These constructs may be an alternative method for providing hydration to the cornea.

## KEY RESEARCH ACCOMPLISHMENTS

- Established that 2 and 3 layer crosslinked amniotic membrane composites are more resistant to enzymatic degradation than monolayer amnion and thus may provide improved protection for the corneal surface of ectropion patient eyes.

- Determined that chemical and photo-initiated crosslinking of amnion proteins is associated with the loss of TGF-β, a cytokine tightly associated with crosslinked proteins in amnion.

- Demonstrated that pre-crosslinked, degradation-resistant amnion can be photobonded to cornea thereby providing an alternative for suturing or a retaining ring to secure these protective membranes to the ocular surface.

- Constructed amnion-covered contact lens and demonstrated that this construct can be photobonded to ex vivo cornea.

**REPORTABLE OUTCOMES:** None during this reporting period.

## CONCLUSION:

In the last year, we have progressed toward our goal of developing materials to protect the eyes of patients with severe periorbital burns and scarring. These patients cannot blink normally or close their eyes (a condition called ectropion), leading to damage to the ocular surface and possibly the need for corneal transplant. Our approach is to stabilize and modify amniotic membrane, a beneficial material for treatment that cannot be used now because it rapidly degrades in these inflamed eyes.

We have now shown that the crosslinked amniotic membrane that was developed in Year 1 can be made even more resistant to enzymatic degradation by forming 2 and 3 layer composites. This is important because the major limitation of the currently available amnion covering is that it degrades rapidly when used to treat cornea of burn patients. Our studies also demonstrated that our crosslinked, degradation-resistant amniotic membrane can be securely sealed to the corneal surface using a sutureless, light-activated technique. This approach is an improvement over the current requirement of a ring on the sclera to hold the amniotic membrane covering in place. In addition, the crosslinked amnion can be cut to fit individual requirements of patients and eliminates the need for sutures that can further damage the cornea. Our study showing that one cytokine, which is closely associated with amnion proteins, is decreased by crosslinking requires follow up studies to assess generality of this observation. We have also demonstrated in principle that an amnion-covered hydrophilic lens can be constructed and sealed to the

cornea. This device would provide hydration to the cornea and thus inhibit damage to the surface for patients who are unable to supply tears by blinking. We are now ready to test these approaches in an animal model for ectropion.

## REFERENCES


1. Bantan-Polak T, Kassai M, Grant KB. A comparison of fluorescamine and naphthalene-2,3-dicarboxaldehyde fluorogenic reagents for microplate-based detection of amino acids. Anal Biochem. 2001 Oct 15;297(2):128-36.
2. Ma DH, Lai JY, Cheng HY, Tsai CC, Yeh LK. Carbodiimide cross-linked amniotic membranes for cultivation of limbal epithelial cells. Biomaterials. 2010 Sep;31(25):6647-58.
3. Dua HS, Gomes JA, King AJ, Maharajan VS. The amniotic membrane in ophthalmology. Surv Ophthalmol. 2004 Jan-Feb;49(1):51-77.
4. Koizumi NJ, Inatomi TJ, Sotozono CJ, Fullwood NJ, Quantock AJ, Kinoshita S. Growth factor mRNA and protein in preserved human amniotic membrane. Curr Eye Res. 2000 Mar;20(3):173-7.
5. Proano CE, Mulroy L, Jones E, Azar DT, Redmond RW, Kochevar IE. Photochemical keratodesmos for bonding corneal incisions. Investigative ophthalmology & visual science. 2004 Jul;45(7):2177-81.